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Human Mismatch Repair Protein hMutLα Is Required to Repair Short Slipped-DNAs of Trinucleotide Repeats*

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Background: Slipped-DNAs are mutagenic intermediates in disease-causing trinucleotide repeat instability; their processing is not well understood.
Results: MutLα is required to repair single short slip-outs and enhances repair of clustered slip-outs.
Conclusion: Aberrant mismatch repair attempts on clustered slip-outs may cause repeat instability.
Significance: This work has determined one of the proteins involved in slipped-DNA repair, which is useful for understanding disease-causing repeat instability.

Mismatch repair (MMR) is required for proper maintenance of the genome by protecting against mutations. The mismatch repair system has also been implicated as a driver of certain mutations, including disease-associated trinucleotide repeat instability. We recently revealed a requirement of hMutSβ in the repair of short slip-outs containing a single CTG repeat unit (1). The involvement of other MMR proteins in short trinucleotide repeat slip-out repair is unknown. Here we show that hMutLα is required for the highly efficient *in vitro* repair of single CTG repeat slip-outs, to the same degree as hMutSβ. HEK293T cell extracts, deficient in hMLH1, are unable to process single-repeat slip-outs, but are functional when complemented with hMutLα. The MMR-deficient hMLH1 mutant, T117M, which has a point mutation proximal to the ATP-binding domain, is defective in slip-out repair, further supporting a requirement for hMLH1 in the processing of short slip-outs and possibly the involvement of hMHL1 ATPase activity. Extracts of hPMS2-deficient HEC-1-A cells, which express hMLH1, hMLH3, and hPMS1, are only functional when complemented with hMutLα, indicating that neither hMutLβ nor hMutLγ is sufficient to repair short slip-outs. The resolution of clustered short slip-outs, which are poorly repaired, was partially dependent upon a functional hMutLα. The joint involvement of hMutSβ and hMutLα suggests that repeat instability may be the result of aberrant outcomes of repair attempts.

Numerous hereditary neurological, neurodegenerative, and neuromuscular diseases including myotonic dystrophy type 1 (DM1)[2] and Huntington disease (HD) are caused by expansions of CTG/CAG trinucleotide repeats (TNRs) (2). In the non-affected population the length of the repeat tract ranges from 5 to 24 repeat units, which are genetically stable. Expanded lengths of 36–6550 repeats can exhibit a high degree of genetic instability, where the number of repeats strongly influences the probability of further mutation. Ongoing TNR expansions in somatic tissues are thought to contribute to disease progression and severity. The mechanism of repeat expansions is not known; however, DNA replication slippage, transcription, and aberrant mismatch repair (MMR) are involved (for review, see Ref. 2).

MMR proteins are required to drive CAG/CTG expansion mutations, which contrasts with their more common role in protecting against mutations (for review, see Ref. 3). In four different transgenic mouse models of CAG/CTG instability (HD and DM1), repeat expansions were stabilized when *Msh2* or *Msh3* was knocked out (4–7). In human cells, transcription-induced CAG repeat contractions were reduced upon knockdown of *MSH2* and *MSH3* (8). Because MMR proteins repair short insertion-deletion loops, it is possible that their mutagenic role in TNR instability is the result of attempts to process CTG/CAG repeat structures.

On a mechanistic level, slipped-DNAs are thought to be mutagenic intermediates of TNR instability. We have shown previously that long slip-outs containing 20 excess repeats can be correctly repaired, escape repair, or undergo error-prone repair depending upon the slip-out sequence and nick location; however, all of these processes are independent of the MMR proteins hMutSα, hMutSβ, hMLH1, and hPMS2 (9). In contrast, isolated short slip-outs of 1–3 repeat units are repaired with very high efficiency, and this repair is hMutSβ-dependent (1). In the presence of multiple clustered short slip-outs, repair efficiency is greatly decreased, likely due to repair interference of adjacent slip-outs. Failed attempts to repair the multiple lesions can be error-prone, indicating a mechanism by which

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[2] The abbreviations used are: DM1, myotonic dystrophy type 1; HD, Huntington disease; MMR, mismatch repair; S-DNA, slipped-DNA; TNR, trinucleotide repeat.

hMutSβ can lead to repeat expansions. The role of MutL homologs in processing slipped-DNAs is currently unknown. In this study, we investigated whether or not hMLH1, hPMS2, hPMS1, and hMLH3 are involved in the repair of short slip-outs. Whereas MutSβ is clearly implicated in causing instability, a role for the MutL complexes has been less apparent.

Following the recognition of unpaired or damaged DNA by either the MutSα (MSH2-MSH6) or MutSβ (MSH2-MSH3) complex, other proteins are recruited to initiate and complete repair. Such factors include the MutL homologs hMutLα (hMLH1-hPMS2), hMutLβ (hMLH1-hPMS1), and hMutLγ (hMLH1-hMLH3), where the latter two complexes play a minor role in mismatch repair relative to hMutLα (10). Studies in yeast models showed that deficiencies of yMLH1 and yPMS1 (hPMS2 homolog in yeast) had no effect on CAG contractions or CTG expansions (11, 12), similar to the absence of an effect by deficiencies of yMSH2, yMSH3, or yMSH6 (13, 14). Yet, in a *Pms2*$^{-/-}$ DM1 mouse model, some rare large repeat contractions were observed, and the rate of expansions was decreased by 50% (15). This partial effect was attributed to the presence of *MLH3* which has some MMR activity (16). In human cells, an *hMLH1* deficiency had no effect on replication-induced repeat contractions (17); however, knockdowns of *MLH1* or *hPMS2* increased transcription-induced CAG contractions (18). A clear role for MutLα, MutLβ, or MutLγ in the processing of slipped-DNAs is lacking. In this study, we investigated whether or not hMLH1, hPMS2, hPMS1, or hMLH3 are involved in the repair of single repeat slip-outs. We found that hMutLα, but not hMutLβ or hMutLγ, is required for the repair of short slip-outs and plays a partial role in the limited repair of clustered short slip-outs, similar to hMutSβ.

EXPERIMENTAL PROCEDURES

Slipped-DNA Substrates—Circular substrates containing a one repeat slip-out were made by hybridizing single-stranded circular DNA with a pure (CAG)47 repeat tract with a double-stranded linearized plasmid containing (CTG)48·(CAG)48 repeats. The site of linearization (EcoRI or HindIII) placed a nick in the CTG strand either 5′ or 3′ to the slip-out. Slipped-DNA (S-DNA) substrates contain an equal number of CTG and CAG repeats (50 repeats). Detailed procedures for making the substrates have been previously described (9).

Cell Lines and Cell Extracts—The HEK293T (293T) cell line was a gift from Dr. G. Plotz. HeLa cells were from the National Cell Culture Center, National Center for Resources, National Institutes of Health. HEC-1-A was from the American Type Culture Collection. Cell extracts were prepared as described (9) from repair-proficient and -deficient cell lines.

Expression Vectors of Wild-type hMLH1 and hPMS2—The expression vectors for the wild-type *hMLH1* (pcDNA3.1-*hMLH1*) and *hPMS2* (pSG5-*hPMS2*) were gifts from Drs. G. Plotz and J. Trojan (19). The pSVwt plasmid for expressing SV-40 T-antigen was a gift from Dr. E. Fanning. The hMLH1 variants (T117M and I219V) were constructed by site-directed mutagenesis of the wild-type vectors using the Site-directed Mutagenesis kit (catalog 200518; Stratagene).

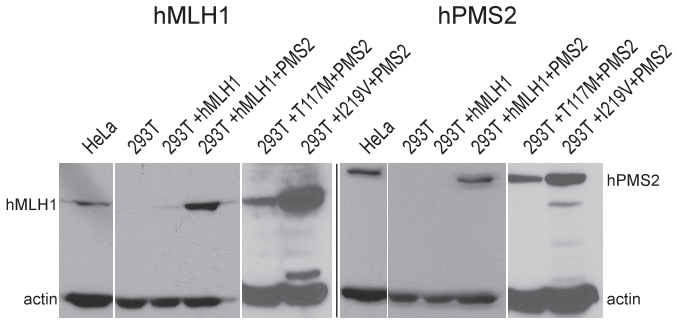

FIGURE 1. **Western blot analysis of the complementation of HEK293T (MLH1-deficient) cell extracts with *hMLH1* and its variants *hPMS2*, and actin.**

Cell Culture and Transfections of Plasmids Containing hMLH1, hMLH1 Variants, and hPMS2 Genes—The 293T cells were grown in DMEM containing 10% FCS and antibiotics. The cells were transfected with plasmids containing *hMLH1* wild-type, *hMLH1* variants, and *hPMS2* genes using FuGENE HD Transfection Reagent (Roche Applied Science). Similarly, HEC-1-A cells lacking *hPMS2* were transfected with SV40 T-antigen plasmid (pSVwt) and *hPMS2* expression plasmid, pSG5-*hPMS2*. The extracts from transfected cells were made as described above.

Western Blot Analysis—Western blots of 293T and HEC-1-A cell extracts were probed with antibodies against hMLH1 (catalog 554073; BD Biosciences), hPMS2 (catalog 556415; BD Biosciences), and actin (catalog 612657; BD Biosciences).

Repair Reactions and Repair Efficiencies—Repair reactions were carried out as described previously (9). Briefly, 22 fmol of circular slipped-substrates was incubated with whole cell extracts, NTPs, dNTPs, creatine kinase, and creatine phosphate for 30 min at 37 °C. The reaction was stopped in 2 mg/ml proteinase K, 2% SDS, 50 mM EDTA, pH 8.0, for 1 h, followed by phenol/chloroform extraction and ethanol precipitation to clean up the reaction. The resulting material was digested with EcoRI/HindIII and run on a 4% polyacrylamide gel for Southern blot analysis. The southern probe used was an EcoRI/HindIII repeat-containing fragment from a related plasmid with (CTG)17·(CAG)17. Repair efficiency is the proportion of radiointensity of the repair product relative to all repeat-containing fragments (ImageQuant Molecular Dynamics v1.2).

RESULTS

HEK293T Cell Extracts Lacking hMLH1 Complemented with hMLH1 and hPMS2—To determine the role of hMLH1 and hPMS2 in the processing of slipped-DNAs formed by TNRs, we used the HEK293T cell line (293T) which is deficient in hMLH1 due to CpG methylation of the *hMLH1* gene promoter and deficient in hPMS2 due to its destabilization in the absence of hMLH1 (16, 19) (Fig. 1). Extracts of 293T cells are deficient in the repair of G-T mismatches, but when co-transfected with wild-type *hMLH1* and *hPMS2* expression constructs, the extracts are MMR-functional (16, 19).

Repair of Short Slip-outs Requires hMutLα—To determine whether the hMutLα complex is required in the repair of short TNR slip-outs, we assessed the repair of a single CTG repeat slip-out in a circular substrate containing a nick located 5′ of

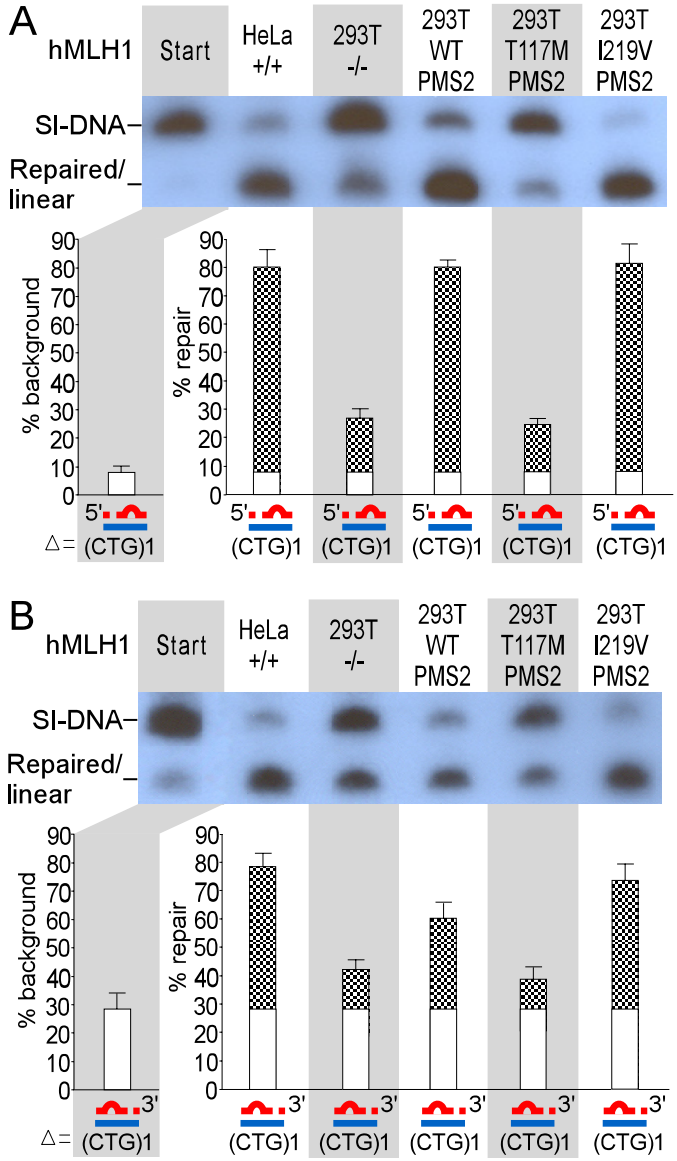

FIGURE 2. **Southern blot analysis of the repair of single CTG slip-out substrates by hMLH1-proficient/-deficient cell extracts.** Repair efficiencies are the percentage of the "repaired/linear" fragment compared with all repeat-containing fragments in the lane ($n = 3$). Graph shows starting background (*white bars*) and repair (*checkered bars*). Substrates contain 5′- (*A*) and 3′-nicks (*B*) on the CTG strand. The repair fragment has (CTG)47·(CAG)47, whereas the slipped heteroduplex SI-DNA fragment has (CTG)48·(CAG)47.

the slip-out. This single-repeat slip-out was processed with MMR-proficient HeLa extracts, hMutLα-deficient 293T extracts, and 293T+*hMLH1*+*hPMS2* extracts. The repair efficiency of 293T was only 18% compared with 73% in HeLa (Fig. 2*A*, compare efficiencies after background correction). This suggested that the inability of the 293T extracts to repair the slip-out was due to the absence of the hMutLα complex. The repair efficiency of 293T cells was fully restored to 73% by transfecting with wild-type *hMLH1* and *hPMS2* genes, indicating a requirement of hMutLα for the highly efficient repair of single repeat slip-outs with a 5′-nick. It is notable that the very high levels of repair of the single repeat slip-out produced by the MMR-proficient HeLa and the hMutLα-corrected 293T extracts exceeded that of a G-T mismatch (one of the best repaired base-base mismatches), which is repaired at 35–40% (1, 20). Thus, the highly efficient repair of single repeat slip-outs requires hMutLα.

Because the requirement of hMutLα was shown to be different for G-T mismatches with 5′- *versus* 3′-nicks (21), we assessed the requirement of hMutLα in the processing of single repeat slip-outs with nicks located 3′ of the slip-out. The repair efficiency of 293T cell extracts was only approximately 12% above background (*see hatched area* in Fig. 2*B*). Repair activity was restored up to 34% over background when complemented with wild-type *hMLH1* and *hPMS2*. Overall, the 3′-nicked substrates were repaired less efficiently than the 5′-nicked substrates, as shown previously (1, 9).

Repair of Short Slip-outs Requires MMR-functional hMLH1 Protein—The ATP-binding and ATPase activity of hMLH1 have been shown to be important for MMR activity (22, 23). We assessed the effect of an hMLH1 point mutation proximal to the ATP-binding site on the repair of single repeat slip-outs. The mutant hMLH1 T117M has been found in tumors of hereditary nonpolyposis colorectal cancer-affected individuals (24–26) and has been shown to be nonfunctional in MMR (19, 27). The T117M mutation maps to a conserved sequence, which is believed to be directly involved in binding and/or hydrolysis of ATP and has been shown to be able to interact with hPMS2 both *in vitro* (28) and in transfected human cells (19, 24). Transfection of the 293T cells with the T117M *hMLH1* and the wild-type *hPMS2* recovered expression of both proteins (Fig. 1). We tested the repair activity of extracts of 293T cells transfected with the T117M *hMLH1* variant and found that this variant could not complement repair (Fig. 2*A*). These results are consistent with the inability of this mutant hMLH1 to restore repair of a G-T mismatch (19, 27) and may suggest that the ATP-binding and/or ATPase activity of hMLH1 is required for functional repair activity of slipped-DNAs.

Similar experiments were performed using substrates with nicks located 3′ of the single repeat CTG slip-out (Fig. 2*B*). Extracts of 293T cells transfected with the ATP binding-defective T117M *hMLH1* did not show restored repair activity. This indicates a requirement of hMLH1 ATP-binding activity for the high levels of single repeat slip-out repair.

Repair of Short Slip-outs Can Be Mediated by a Functional Rare Variant of hMLH1—Because there have been numerous suggestions in the literature that polymorphic variants of DNA repair proteins may have functional differences in their repair activities, we assessed repair using a rare functional polymorphic variant of hMLH1. The I219V hMLH1 protein has an allele frequency ranging from 3 to 36% depending upon the studied population (19, 29–33). Although MMR-competent (19), the I219V variant has been significantly associated with radiation-induced rectal or bladder toxicity (34) and increased risk of breast cancer (35) and childhood acute lymphoblastic leukemia (36), indicating that it may be functionally different from other hMLH1 variants. Studies have shown the I219V site can have variable risks toward a variety of cancers including ovarian (37), lung (38), breast (39), ulcerative cancer (40), and prostate cancer (41).

The I219V hMLH1 variant was shown to interact with wild-type hPMS2 and hEXO1 at levels equal to or greater than the

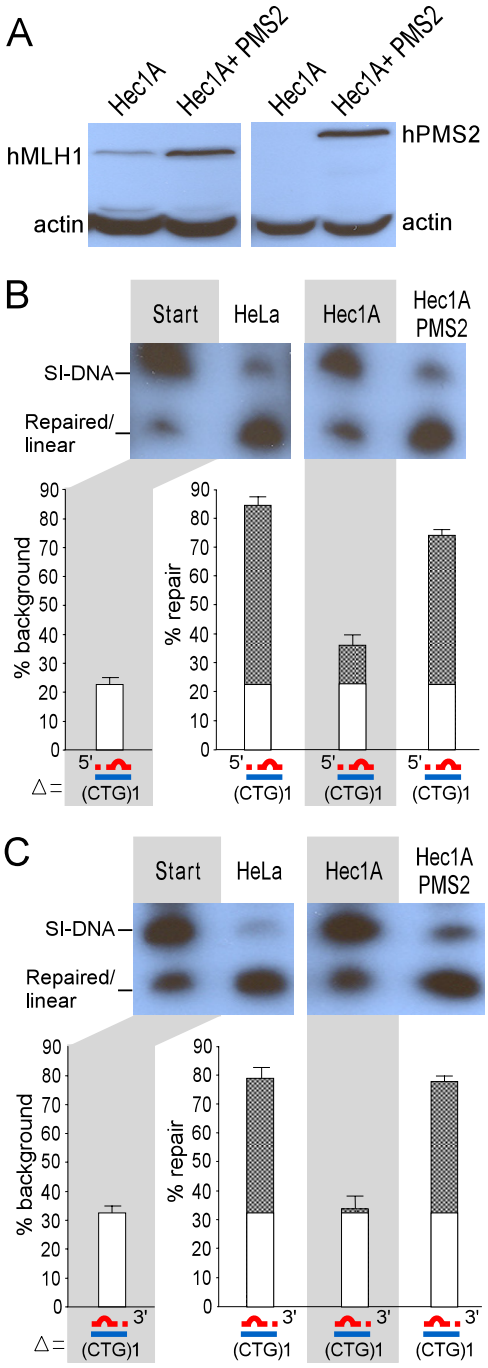

FIGURE 3. **hPMS2 is required to repair short slipped-DNAs.** *A*, Western blot analysis of the complementation of HEC-1-A (hPMS2-deficient, proficient in both hMutLβ and hMutLγ (16, 43, 44)) cell extracts. *B* and *C*, Southern blot analysis of the repair of single CTG slip-out substrates by hPMS2-proficient/-deficient HEC-1-A cell extracts. Substrates contain 5′- (*B*) or 3′-nicks (*C*). The repair fragment has (CTG)47(CAG)47, whereas the S-DNA fragment has (CTG)48(CAG)47.

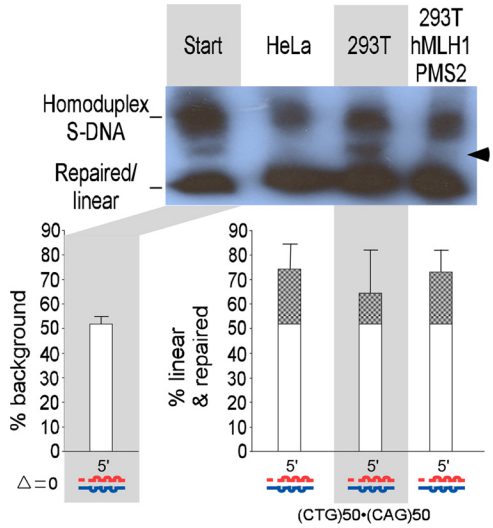

FIGURE 4. **Southern blot analysis of the repair of multiple clustered slip-outs (S-DNAs) by hMLH1-proficient HeLa extracts and hMLH1-deficient and complemented 293T cell extracts.** *Arrowhead* indicates S-DNA which has hMLH1-dependent repair. The repair fragment has perfectly paired (CTG)50•(CAG)50, whereas the homoduplex slipped S-DNA fragments have multiple clustered short slip-outs on either strand of the (CTG)50•(CAG)50 tract.

wild-type *hMLH1* (42). Extracts from cells transfected with *hMLH1* constructs carrying I219V mutation completely restored the repair activity (Fig. 2*A*). Similarly, the I219V variant could complement the repair efficiency of the 3′-nicked single repeat slipped substrate, where the 3′-nicked substrate was less efficiently repaired than the 5′-nicked substrate (Fig. 3*B*). Thus, the level of activity of the I219V MLH1 variant did not appear to differ significantly from the more common wild-type form of MLH1 in MutLα, similar to what has been seen previously for G-T mismatch repair (24).

Repair of Short Slip-outs Requires a Functional hPMS2 Protein—Although the above results reveal a requirement of hMutLα for the repair of single repeat slip-outs, it is unclear whether this represents a requirement of hMLH1 or hPMS2. To distinguish between these two proteins, we used the HEC-1-A cell line, which is genetically deficient in both *hPMS2* (43, 44) and *hMSH6* (16, 44, 45). It contains a nonsense mutation in the *hPMS2* gene, which results in premature termination and a truncated hPMS2 protein. We have characterized *hMLH1* and *hPMS2* expression levels in HEC-1-A and found that, as expected, hMLH1 is present and hPMS2 is absent (Fig. 3*A*). Thus, we were able to use HEC-1-A extracts to study the role of hPMS2 in the repair of short slip-outs containing either 5′- or 3′-nicks. For 5′-nicked substrates, the repair efficiency of HEC-1-A extracts is only 14% (Fig. 3*B*), which is comparable with the background repair of these substrates by hMSH2-deficient LoVo extracts (1, 9). In contrast, MMR-competent HeLa extracts yielded 75% repair of the single repeat slip-out. When the HEC-1-A cell line is transfected with a wild-type *hPMS2* expression construct, the repair efficiency of the extract is fully restored (Fig. 3*B*). This clearly shows that hPMS2 is required for the repair of short repeat slip-outs. The requirement for hPMS2 was seen for both 5′- and 3′-nicked substrates, with 5′-nicked substrates being repaired more efficiently than 3′-nicked substrates (Fig. 3, *B* and *C*).

Multiple Clustered Short Slip-outs, S-DNAs, Are Poorly Repaired, and This Is Further Reduced in the Absence of hMutLα—Homoduplex S-DNAs harbor a series of clustered small slip-outs, each containing 1–3 repeat units (46). For example, in S-DNA of (CTG)50•(CAG)50 the majority of molecules contain up to 62 slip-outs distributed evenly between both strands along the length of the repeat tract (1, 46). The role of hMLH1 in the repair of clustered short slip-outs is not

known. We showed previously that S-DNA substrates were very poorly repaired by MMR-proficient HeLa extracts, yielding only 10–26% repair (1). This limited amount of S-DNA repair was further reduced to 6–9% when hMSH2-deficient LoVo extracts were used. Here, we tested the effect of an hMLH1-deficiency upon the repair of S-DNA. The starting circular-nicked S-DNA harbored both slipped and fully duplexed DNAs, where the latter was background (Fig. 4, *left lanes* and *hatched bars*). The high background level of fully duplexed material is unavoidable because this is simply the level at which complementary (CTG)50 and (CAG)50 repeat strands come together as fully paired; it is inseparable from S-DNAs as circular duplexes (47). This background does not hinder repair assessment (1). S-DNA substrates were incubated with HeLa, 293T, or 293T+*hMLH1*+*hPMS2* cell extracts. These all yielded very low levels of repair (Fig. 4, *hatched bars*). However, the level of repair mediated by the 293T extracts (13%) was somewhat less than that produced by the 293T+*hMLH1*+*hPMS2* (20%), which was similar to that yielded by the HeLa extracts (22%). Whereas the presence of multiple slip-outs clustered on either strand greatly inhibited repair, it was not altogether ablated. The presence or absence of hMLH1 or hPMS2 partially enhanced the repair of S-DNA (Fig. 4). Interestingly, the repair of one species of S-DNA was completely dependent upon the presence of MLH1 (Fig. 4, *arrowhead*).

DISCUSSION

Most mammalian studies of mismatch repair in TNR instability have focused on the roles of MSH2 and MSH3, with very few having investigated a role for MLH1 and its binding partners: PMS2, PMS1, and MLH3 (for review, see Ref. 2). In MMR, the MutS and MutL homologs are collectively needed to repair mismatched bases and insertion-deletion loops in an orchestrated manner. Previous studies have found that repair of a heteroduplex with a single CTG insert (not in the context of an expanded repeat tract) by MutSα-proficient HCT116 (H6) cell extracts was defective before complementation with purified hMutLα (48, 49). In contrast to repair processes, the mutagenic event of TNR instability may not involve the joint actions of MMR proteins. In transgenic mouse models of HD and DM1, deficiencies in *Msh2* or *Msh3* led to somatic repeat stabilization and germ line contractions; however, a PMS2 deficiency in a DM1 mouse model only decreased expansions by 50% (15). In human cell line assays focused upon TNR contractions, transcription-induced CAG contractions were decreased by *MSH2* and *MSH3* knockdown, and yet contractions were increased with a knockdown of *MLH1* or *PMS2* (8, 18). Previously, we found that the highly efficient repair of isolated short slip-outs required MSH2/MSH3, but not MSH6 (1); in this study we determined that MLH1 and PMS2 are also required for this repair activity. Additionally, MLH1 must be MMR-functional to repair short slip-outs because an ATPase-mutant was not able to perform repair successfully. This is similar to the *in vivo* ATPase requirement of MSH2 for instability in mice (53). Our results predict that in mouse models of TNR instability MLH1 will play as crucial a role as MSH2 and MSH3.

In eukaryotic systems there are two alternate MutL homologs other than MutLα (MLH1-PMS2): MutLβ (MLH1-PMS1) and MutLγ (MLH1-MLH3). Analysis of several human cancer cell lines showed that hPMS1 is 10 times less abundant than hPMS2 (22) and hMLH3 levels are 60 times below those of hPMS2 (16). In yeast it has been suggested that yMLH3 is involved in the repair of the same types of insertion-deletion loops as yMSH3, as *mlh3* and *msh3* single mutant strains display a mutator phenotype similar to double mutant strains (50, 51). This could be extrapolated to suggest that MLH3 would be required for repair of short TNR slip-outs. hMutLβ and hMutLγ may have minor roles in MMR (*Pms1*- and *Mlh3*-knock-out mice display microsatellite instability, Ref. 52), but they are much less active in MMR than hMutLα (10). The 293T cell line used in this study lacks hMLH1, hPMS2, and hMLH3 proteins, and it has been observed previously that overexpression of hMutLα or hMutLγ in these cells can partially restore the repair of G-T mismatches (16). However, hMutLγ had to be added at very high nonphysiological levels, and the repair was still much less efficient than with expression of hMutLα. Using the HEC-1-A cell line (which expresses hMLH1, hPMS1, and hMLH3, but not hPMS2 or hMSH6 (16, 43, 44)), our short slip-out repair assay allowed us to determine whether hMLH3 or hPMS1 is required for short slip-out repair. A deficiency in hMSH6 is not detrimental to the repair of short slip-outs, as we have shown previously that MSH3, but not MSH6, is required for this processing. At endogenous levels, neither hMLH3 nor hPMS1 appeared to act as a back-up for hPMS2 in the repair of short slip-outs because their repair was as poor by the HEC-1-A extracts as for the hMutLα-deficient 293T extracts; however, we cannot exclude the possibility that inclusion of excess nonphysiological levels (as in cancer lines) of hMutLγ could restore repair activity.

In summary, the MMR MutS and MutL homologs are required to repair mismatched bases and insertion-deletion loops in an orchestrated manner. Our finding that human MutSβ and MutLα are equally required to repair short repeat slip-outs supports the idea of mutagenic outcomes of repair attempts leading to ongoing TNR instability. Our results predict that in mouse models of TNR instability, and in affected humans, MLH1 will play as crucial a role as MSH2 and MSH3.

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